Production of Human Paraoxonase I (huPON1) In *E. coli* With Periplasmic Expression and Chaperone Co-expression

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by

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Abstract

Paraoxonase-1 (PON1) catalyzes the hydrolysis of organophosphorous (OP) nerve agents and has potential as a therapeutic agent for OP poisoning. We are working on several aspects of PON1, such as understanding the catalytic residues involved in PON1, along with improving its stability and drug-like properties. Specifically, I investigated the expression of human paraoxonase 1 (huPON1) in the periplasm, the space between the plasma membrane and the peptidoglycan wall of *E. coli*, using Maltose Binding Protein (MBP) as a solubility enhancer of our recombinant protein. Most of the initial work was done using a chimeric (recombinant) rabbit homolog (rePON1 or G2E6) that expresses well in *E. coli*. However, in order to utilize PON1 therapeutically, it likely needs to be in fully humanized form. The major challenge here is the solubility and expression of human paraoxonase in bacterial systems such as *E. coli.* MBP fusion was used to improve the solubility of huPON1 in *E. coli.* The periplasmic expression is advantageous because it promotes disulfide formation; since huPON1 contains a disulfide bond, this strategy can potentially improve its soluble expression. This strategy has proven useful for other difficult-to-express proteins with disulfide bonds, such as antibody fragments. A well-expressed, stable human protein within a bacterial system will help lead us closer to finding a sufficient treatment against organophosphate poisoning.

We also utilized the co-expression of molecular chaperones. One of the principal problems of expressing protein in bacterial systems is the aggregation of proteins through their interactions among one another during their folding process. This can result in protein degradation and improper native structure. Just as periplasmic expression enhances proper protein folding, the application of chaperones sets out to catalyze protein folding within the

cytoplasm of *E. coli*. The co-expression of huPON1 with chaperone systems such as GroEL-GroES and DnaK/DnaJ/GrpE could prove to be very useful for correct protein folding.

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Organophosphate Background

Organophosphorous antiacetylcholinesterases (OP agents) such as Sarin, VX, VR, and paraoxon are highly toxic to humans.[1,2] These nerve agents inhibit acetylcholinesterase at the synapse of neuromuscular junctions causing acetylcholine concentration to increase within the cleft.[3] The buildup of acetylcholine leads to continuous firing of neurons and produces intramuscular convulsions.[4] The known toxicity has proven lethal in chemical warfare by terrorist groups against civilian populations.[2] There has been minimal success in rapid treatment to date. All treatments for OP poisoning thus far are given post exposure.[3] Atropine, an antagonist at the muscarinic acetylcholine receptor, is among the treatments. Yet, reaching the OP agent before it arrives at the synapse could more efficient than post-exposure treatments.

Our goal is to find a human protein capable of hydrolyzing organophosphates before and after exposure. However, the ability to produce a catalytic protein in massive quantity along with having economic practicality is among the top challenges for creating a new treatment.[5] Furthermore, high catalytic activity, optimal half-life, immunotolerance, and the ability to turn over a diverse range of OPs are some of the required properties the therapeutic protein must possess. Prokaryotic systems are ideal for manipulating specific recombinant genes with the over-expression of a target protein. Specifically, *E. coli* has proven to be extremely versatile in the expression of recombinant human protein.

Therapeutic Agent

The target proteins known to have potential therapeutic activity against nerve agents are the human organophosphorous acid anhydride hydrolases (OPAHs), including human paraoxonase (huPON).[6] PON1 and PON3 of the PON family can be found on high-density lipoprotein (HDL).[7] Furthermore, the lactonase and anti-atherogenic activity of PON1 makes it

the protein of interest.[1] The crystal structure has not yet been determined for huPON1, but it is available for recombinant PON1 and is described as a six-bladed β-propeller with each blade containing four strands. There is also a site that is also thought to be involved in HDL binding. PON1 has three cysteine residues, with two of them (Cys42 and Cys353) forming a disulfide bridge. Two calcium ions are located in the central part of the tunnel in the propeller. One is believed to play structural role and the other a catalytic one.[3]

The catalytic residues involved in PON1 can help us understand several aspects of its mechanism, along with improving its stability and drug-like properties. However, in order to utilize PON1 therapeutically, it needs to be in fully humanized form. The major challenge here is the solubility and expression of human paraoxonase in bacterial systems such as *E. coli.* Another challenge is creating a stable and well-folded protein (huPON1) capable of turning over organophosphates at a formidable rate.

Recently, Clement Furlong and others have reported the first *E. coli* expression and purification of huPON1, along with other variants of PON1.[8] However, their yields are very low. They have reported 5.5 mg of 90% pure protein from 12 L of fermentation. Since cultures can be fermented to densities 10X as high as in a shake flask, that would correspond to 0.046 mg per liter of shake flask culture. This validates the challenge of expressing and purifying soluble human protein within a bacterial system.

The approaches I have taken to combat these issues include using Maltose Binding Protein as a solubility enhancer tag, periplasmic expression to promote disulfide formation, and the utilization of chaperones to increase native folding of huPON1.

MBP Fusion

Maltose Binding Protein (MBP) will be employed as a solubility enhancer tag for improved solubility of rePON1 and huPON1. MBP fusion has been shown to improve the solubility of many proteins that were insoluble in *E. coli.*[9] Waugh and co-workers demonstrated this by directly comparing three different solubility tags; *E. coli* His-patch thioredoxin (TRX), *Schistosoma japonicum* glutathione S-transferase (GST), and *E. coli* maltose-binding protein (MBP). MBP was fused to six different polypeptides (TIMP, p16, E6, CATΔ9, GFP, TEV) that are commonly insoluble in *E. coli.* All fusion proteins were expressed at a high level in BL21 (DE3) cells, revealing yields from 15% to 35% of total intracellular protein. Based on comparisons, MBP fused proteins were shown to have a greater overall solubility than TRX and GST fused proteins. The enhanced solubility of the formerly insoluble proteins is an indication it may be useful for increasing the problem of solubility of huPON1 in *E. coli*. Furthermore, the MBP tag can be easily utilized along with other strategies such as expression within the periplasm of *E. coli*.

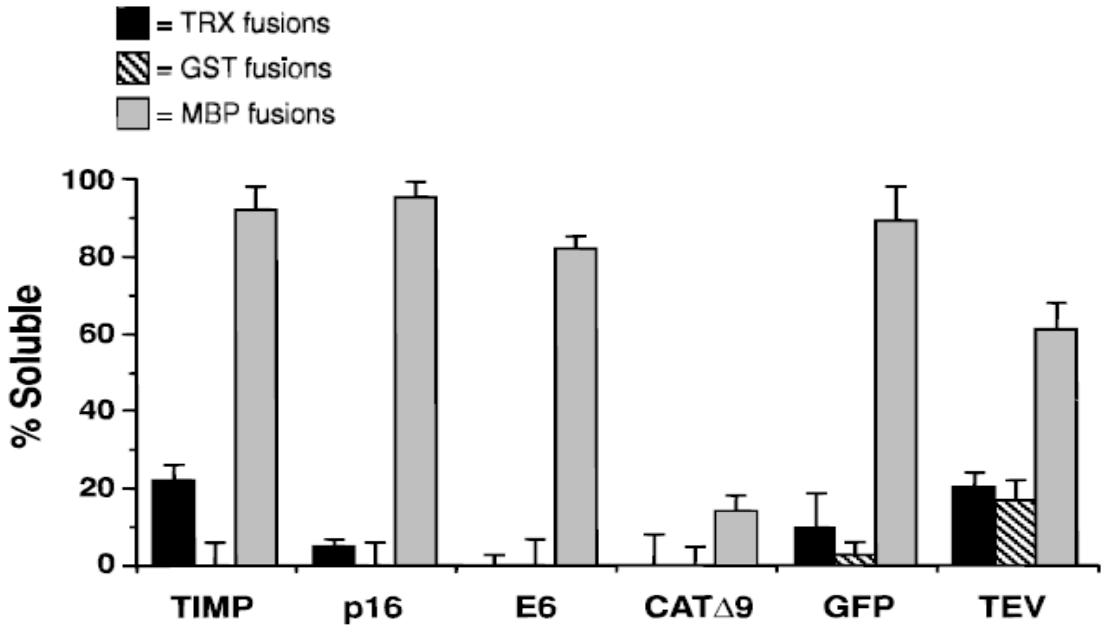


Figure 1. Comparison between protein solublility fusion tags. MBP-fusions shows most improved protein solubility based on SDS-PAGE gels in each of six polypeptides.[9]

Periplasmic Expression

Periplasmic expression is advantageous because it contains a much less reducing environment in comparison to the cytoplasm. Since huPON1 contains the disulfide bond between Cys43 and Cys353, this strategy can potentially improve the soluble expression of the protein by creating a more conducive environment for disulfide formation. Periplasmic expression has proven useful for other difficult-to-express proteins with disulfide bonds, such as antibody fragments.[10] For example, fragment antigen binding or single chain fragment variable is a disulfide-bonded protein with therapeutic importance. The secretion of the fragment antigen binding chains into the oxidizing environment of the periplasm has produced more stable and native folded protein. Another example includes the purification of RNaseA fused with His_6MBP containing the signal peptide for periplasmic export.[11] As shown below, RNaseA shows large amounts of activity once exported and expressed in the periplasm. This approach could support the formation of a stable huPON1.

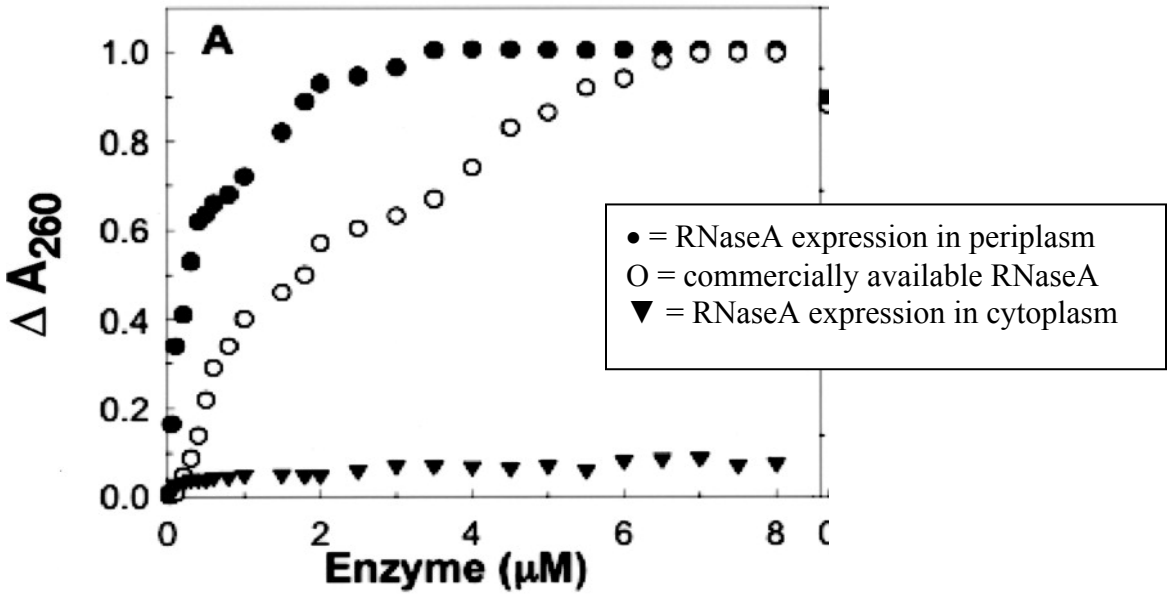


Figure 2. Enzymatic activity of RnaseA fusion proteins. Expression in the periplasm showed the most change in absorbance and therefore the most activity.[11]

Chaperones

Molecular chaperones assist and avert the misfolding of a protein due to adverse conditions within the cell.[12] Early researchers believed inclusion bodies or protein aggregates to be unfolded protein and therefore were unaffected by proteases or chaperones.[13] The over-expression system in *E. coli* is more prone to produce protein aggregates and the accumulation of protein in the cytoplasm. This can be toxic to the cell and thus there is less soluble protein.[14] However, Carrio and Villaverde prove that protein aggregation due to over-expression of targeted genes can be reversed in vivo. Carrio and Villaverde did so by stopping protein synthesis of a β-galactosidase fusion protein, therefore preventing over-expression, and observed an increase in β-galactosidase activity and total soluble protein.[8] Much research has been dedicated to finding ways of taking aggregated proteins and allowing them to re-fold into native conformation. Recent approach is the co-expression of target recombinant genes with chaperone systems.[15] Co-expression systems using chaperones have been confirmed in improving the yield of soluble protein in both bacterial and eukaryotic cells. This is done by working against kinetic dead-end traps and helping the wrongly folded (aggregated) protein to go back and reach its thermodynamic minimum.[16] Also, chaperones can help to stabilize interactive surfaces that are capable of interacting with other folding molecules.

The use of chaperone systems including DnaJ/DnaK/GrpE and GroEL/GroES has been a hot topic of study. Ario and Valeria de Marco confirm that co-transformation of different combinations of chaperones with target recombinant proteins in independent plasmids can increase the yield of soluble protein.[15] The target recombinant proteins (GTR1/Xklp3B) exhibit increased solubility based on varying chaperone combinations according to Figure 3.

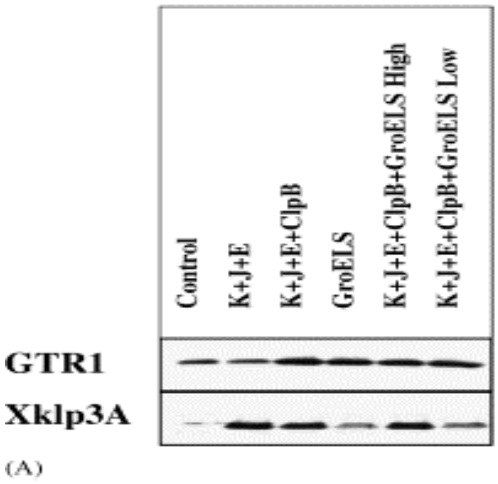


Figure 3. Amount of soluble target protein GTR1/Xklp3B with various chaperone co-expression from SDS-PAGE gel. Each combination (K+J+E = DnaK/DnaJ/GrpE; ClpB = caseinolytic peptidase B; GroELS = GroEL/GroES) shows improved protein solubility in comparison to the control.[15]

The studies have recently moved to whether or not specific over-expressed chaperones can be applied in a general way to various human proteins. One specific study focused on the use of DnaJ/DnaK/GrpE and GroEL/GroES chaperones and their effect on human protein kinases.[17] They observed protein kinases unaffected by chaperone over-expression, protein kinases that were aggregated although showed increased solubility, and lastly kinases that were both aggregated and folded with increased solubility with chaperone over-expression. This demonstrates that specific proteins were each affected differently in a case-specific way for each chaperone system.

Another experiment specifically relates to a human protein having increased solubility in *E.coli*—and the analogous effect is sought for huPON1.[18] Authors first tried expressing recombinant Endostatin (20 kDa), an anti-angiogenic protein and inhibitor of tumor growth, but nearly all was insoluble and formed inactive inclusion bodies. The gels below show the insoluble protein on gel one (A), then, the solubilized protein on gel two (B) with the co-expression of chaperones.

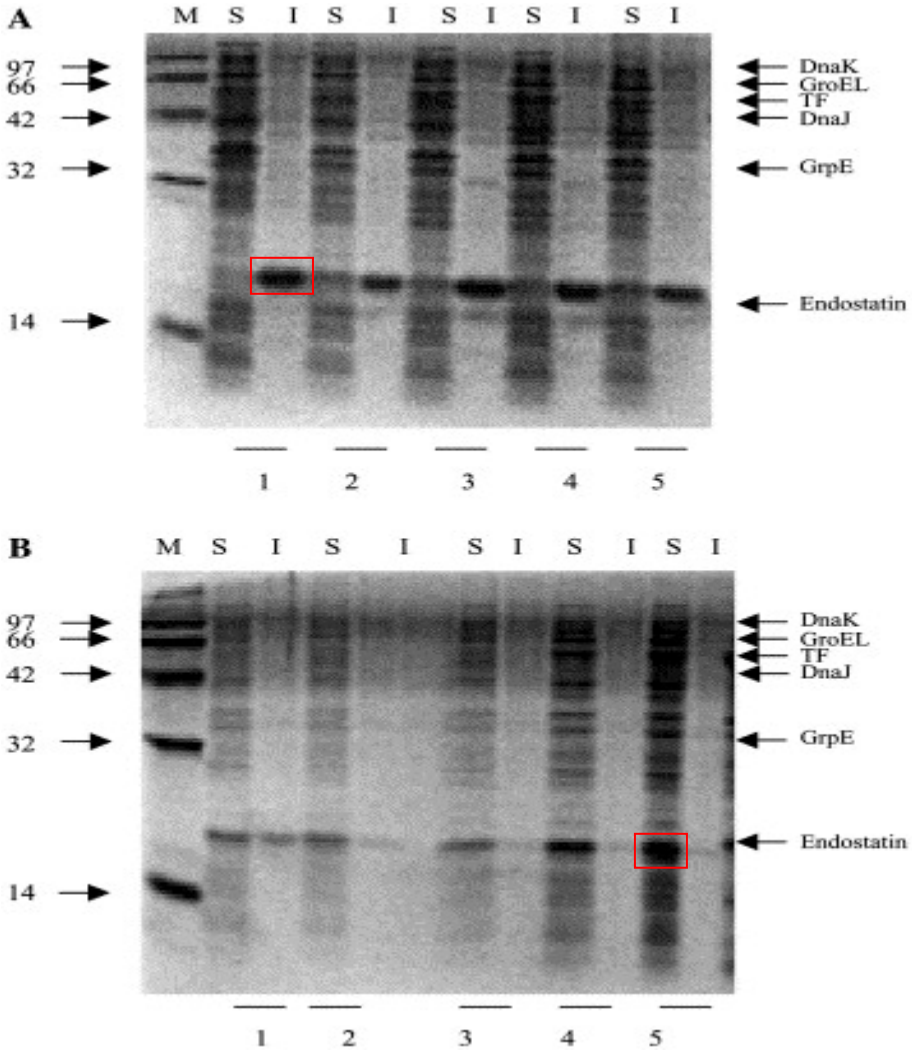


Figure 4. Co-expression of chaperones with recombinant Endostatin separated into soluble (S) and insoluble (I) fractions. Expressions done at 37 °C (A) and 16 °C (B). (B) shows improved soluble protein fraction expressed at 16 °C.[18]

DnaK, DnaJ, and GrpE function together within the cell to catalyze the folding of various proteins. DnaK has an N-terminal ATPase (44 kDa) domain and C-terminal peptide-bonding domain (27 kDa). It functions by binding and releasing proteins using ATP.[19] It does so by recognizing exposed hydrophobic side chains. Three *E. coli* J-proteins (DnaJ, DjlA and CbpA) interact with DnaK and accelerates its ATPase activity. DnaJ (41 kDa) is the major J-protein and

is encoded on the same gene with DnaK. DnaJ has four distinct domains—an N-terminal J-domain that binds with DnaK, a glycine/phenylalanine domain, a zinc-finger domain to help with ATPase activity of DnaK and a C-terminal domain that helps recruit hydrophobic nascent chains to DnaK. Finally, GrpE acts as a nucleotide exchange factor, inducing the release of ADP from DnaK. Figure 5 shows the mechanism of the DnaK/DnaJ/GrpE chaperones.

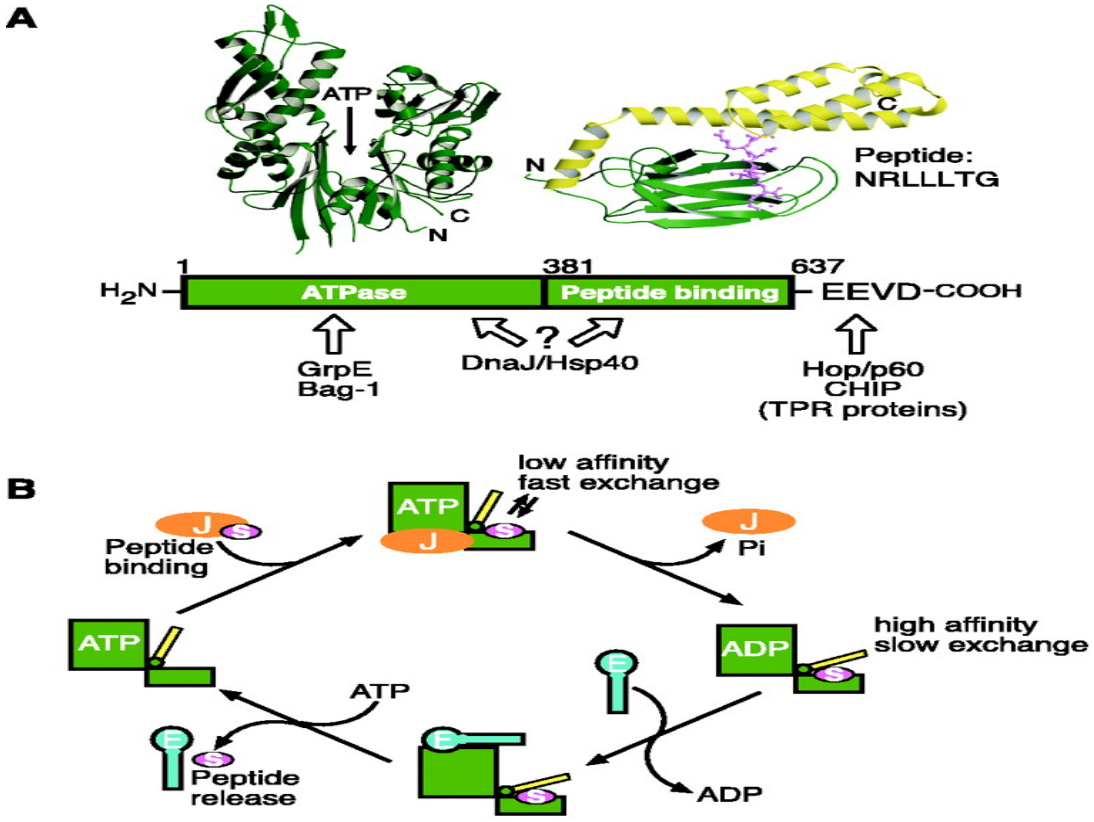


Figure 5. Mechanism of DnaK/DnaJ/GrpE chaperone system.[19]

GroEL/GroES also function together by catalyzing and increasing the folding rate of protein. In GroEL, two heptameric rings of identical 57 kDa subunits stacked back-to-back. It consists of an equatorial domain with an ATP-binding site which is connected through an intermediate hinge-like domain to the apical domain. A cylinder is created from the hinge-like to the apical domain exposing hydrophobic residues toward the ring cavity. GroES (10 kDa) is a

homoheptameric ring that is regulated by GroEL ATPase. GroES cycles on and off of the GroEL cylinder. Proteins up to 60 kDa can be encapsulated and can fold in the GroEL-GroES "cage." Figure 6 shows the mechanism of the GroEL/GroES chaperones.

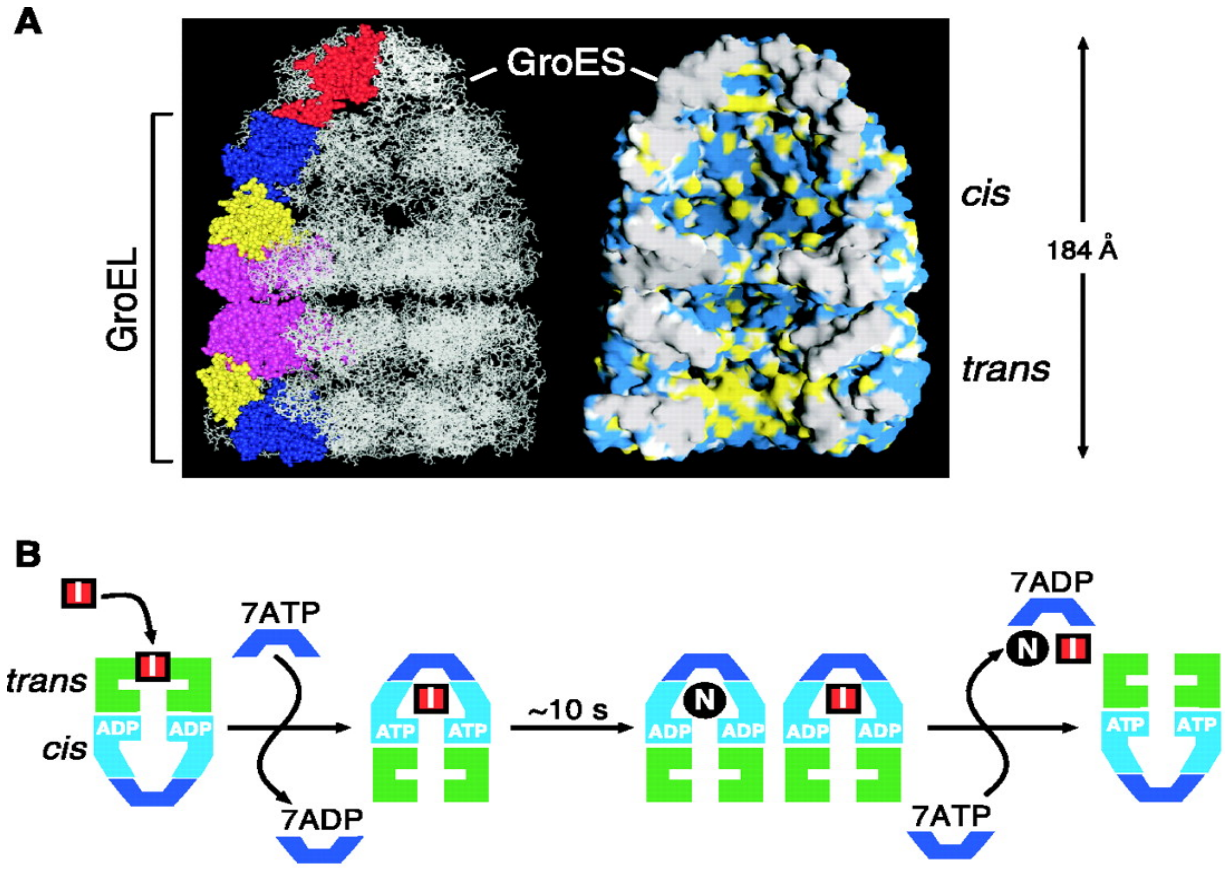


Figure 6. Mechanism of GroEL/GroES complex.[19]

Objectives

Expression and transport of G2E6 and huPON1 into periplasm

The cysteines at positions 42 and 353 are the target of folding for the native huPON1 structure in the periplasm. The periplasm is situated between the inner cytoplasmic membrane and outer membrane or the space between the plasma membrane and the peptidoglycan wall in *E. coli*. Disulfide formation could be enhanced due to the oxidizing nature of the periplasm.

Formation of a protein containing disulfides within the cytoplasm is more difficult due to the reducing environment in *E. coli.* Yet, rePON1 has demonstrated both activity and stability when expressed with *E. coli*, while huPON1 has produced large amounts of protein but was not soluble or active. Therefore, the possibility of transporting huPON1 synthesized in the reducing environment of the cytoplasm to the less reducing environment of the periplasm is a valuable strategy.

Increase solubility and activity of huPON1 in E. coli

Recombinant proteins expressed within *E.coli* are often able to produce sufficient yields, but are still difficult to solubilize and keep a certain amount of activity from the protein to turn over substrate.[9] In our case, huPON1 has reported high expression levels but with minimal solubility and no activity from purified protein. If the end result is to produce a sufficient amount of the therapeutic protein, then, there must be a large enough production of soluble protein in order to properly vaccinate a patient exposed to OP toxins. Additionally, the use of several strategies such as utilizing Maltose Binding Protein as a solubility tag along with bacterial chaperones could potentially help this problem. Both strategies of maltose binding protein and molecular chaperones are capable of guiding the target protein in unfavorable conditions that could potentially keep the protein from forming into its native structure. The ultimate goal is to provide a proficient, stabilizing environment for the protein to be able to fold into its native state.

Methods

Creation of a periplasmic expression vector for G2E6 and huPON1

In order to express our target protein within the periplasm, a vector containing a signal sequence is required for transportation of our target protein (G2E6, huPON1) to the periplasm of

E. coli. The strategy was to use the periplasmic signal sequence from David Waugh's pDEST-periHisMBP vector. pDEST-periHisMBP was utilized for the signal peptide MBP, His6-tag, and MBP sequence. The sequence was then inserted between the NdeI (344) and KpnI (458) sites in pET32b-G2E6 already containing recombinant PON1. The thioredoxin (trx) solubility tag was therefore replaced by MBP in pET32-G2E6.

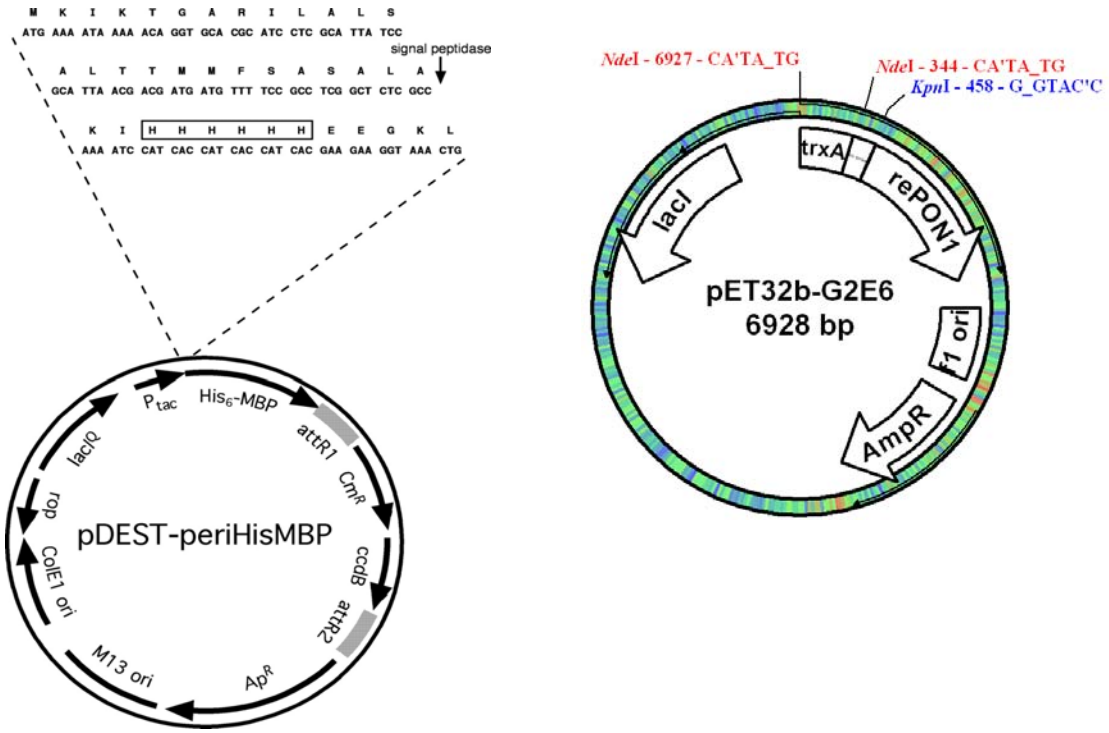


Figure 7. David Waugh's periplasmic expression vector, pDEST-periHisMBP.[11]

The signal sequence for the periplasm was extracted using PCR reactions. Forward and reverse primers were created to use on pDEST-periHisMBP to facilitate the amplification of the signal peptide, His-tag, and MBP.

Table 1. *Primers for pDEST-periHisMBP signal sequence*

Primers	Sequences in 5' to 3'
Forward	AATAAT AATCATATGAAAATAAAAACAGGTGCA NdeI *Signal Sequence*
Reverse	ATTATTATTGGTACCGCCCTGAAAATACAGGTTTTCCTCGAT KpnI TEV CGTTTCTCGTTCAGCTTTTTTGTACAAACTTGTGATCGAA

Finally, the 1200 base pair band of the amplified signal sequence was inserted into the pET32b-G2E6 vector. The signal sequence was ligated into pET32b-G2E6 at 16 °C overnight. Next, the reaction was transformed into DH10B electrocompetent cells and the DNA was recovered through minipreps. The vector was expressed by transformation into BL21(DE3) to facilitate the production of G2E6 as an MBP-fusion protein. The expression was done with 100 mL of 2YT media containing 100 μg/mL ampicillin and 0.11 g/L and grown at 37 °C. Then, it was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a 0.1 mM final concentration after the log phase was reached (A_{600} = 0.8) and placed at 30 °C for 4 hrs. The cells were then pelleted by centrifugation and placed at –80 °C. For purification of the fused protein, David Waugh's osmotic shock procedure was employed.[9] Frozen pellet was resuspended in 30 mM Tris-HCl (pH 7.3) with 20% sucrose (80 mL of solution/gram of cell weight recovered). Then, EDTA (1 mM final concentration) was added and cells were pelleted by centrifugation. Next, the pelleted cells were resuspended in ice-cold water (40 mL/gram pelleted). This solution was mixed at 4 °C for 10 minutes and cells were centrifuged once again. The osmotic shock fluid was considered the supernatant after this centrifugaton. Ni-NTA agarose (0.3 mL of 50 percent) was equilibrated

with lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$, 10% Glycerol, 0.1% Tergitol) and used to capture and bind to our fusion protein. The buffers used in the purification of G2E6 are shown in Table 2.

Table 2. *Buffers for purification of G2E6 (periplasmic)*

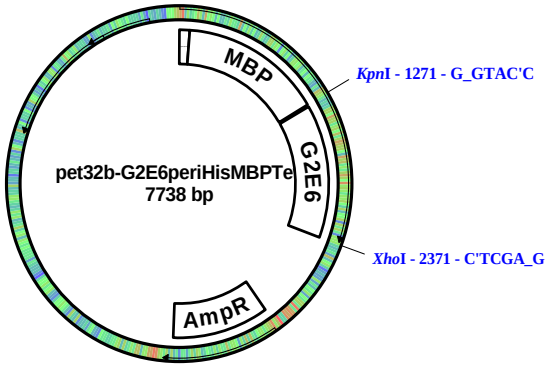
All buffers contain 50mM Tris-HCl pH 8.0, 50mM NaCl, 1mM $CaCl_2$, 10% Glycerol, 0.1%Tergitol		
Buffer Name	Amount of Imidazole	Amount Used
Wash 1	5 mM	5 mL
Wash 2	10 mM	5 mL
Wash 3	25 mM	2 mL
Elution 1	125 mM	1 mL
Elution 2	125 mM	2 mL

Purified G2E6 was tested for activity against the pesticide paraoxon. Paraoxon hydrolysis activity was determined by using paraoxon (diethyl ρ-nitrophenyl phosphate) in 50 mM Tris, 1 mM $CaCl_2$ buffer at pH 7.4. Initial rates of ρ-nitrophenoxide formation (ε=18290 $M^{-1}cm^{-1}$) were followed at 412 nm absorbance for 10 minutes. The eluted protein, G2E6, was verified on a 12.5% SDS-PAGE gel.

Multiple experiments followed with the same type of expression only in a larger scale (1 L) and varying purification conditions. We also attempted a new technique for recovery of periplasmic proteins came from French and Moore.[20] One of the first changes was the resuspension buffer consisting of 200 mM Tris-HCl pH 7.5 and 20% sucrose. Another variation was the addition of lysozyme (500 μg/mL) to the resuspension buffer. As a control, the pelleted cells (1 L culture expressions) were split into two—one resuspended in buffer containing

lysozyme and one without. All other conditions were kept the same, including the wash and elution buffer contents (scaled up for 1 L culture).

The gene from huPON1 was cloned into the new construct, pET32-periHisMBP. huPON1 from pET32b-huPON1trx was amplified using PCR. Then, huPON1 was ligated



Primer	Sequence 5'to 3'
For huPON1	AATAATGGTACCGGTTCTTCTGGAGCC KpnI ATGGCGAAGCTGATTGCG
Rev huPON1	ATTATTCTCGAGTGCGGCCGCTTAGA XhoI GCTCAC

between the *Kpn* I and *Xho* I sites. Once huPON1 was cloned into the construct, it was transformed into BL21(DE3) electrocompetent cells. Each step was followed identically in regards to expression and purification of huPON1 as to G2E6. The initial experiment used 1 L 2YT culture expressions, and protein was purified using lysozyme resuspension buffer. Again, another non-lysozyme resuspension buffer was used on the other half of the frozen pellet. Purified huPON1 was tested for activity against the anti cholinesterase inhibitor, paraoxon. Paraoxon hydrolysis activity was done with the same conditions used above. The eluted protein, huPON1, was verified on a 12.5% SDS-PAGE gel. Finally, the experiment was repeated to confirm the results.

Co-expression of huPON1 with multiple chaperone systems

Plasmids containing molecular chaperones were obtained from Takara Bio Inc. (Japan) and include pG-KJE8, pGro7, pKJE7, pG-Tf2, and pTf16. Plasmid details are included in table 3.[20]

Table 3. *Chaperone plasmids acquired from Takara Bio Inc. (Japan)*

No.	Plasmid	Chaperone	Promoter	Inducer	Resistant Marker
1	pG-KJE8	dnaK-dnaJ-grpE groES-groEL	*araB* *Pzt1*	L-Arabinose Tetracyclin	Cm
2	pGro7	groES-groEL	*araB*	L-Arabinose	Cm
3	pKJE7	dnaK-dnaJ-grpE	*araB*	L-Arabinose	Cm
4	pG-Tf2	groES-groEL-tig	*Pzt1*	Tetracyclin	Cm
5	pTf16	tig (Trigger)	*araB*	L-Arabinose	Cm

Each plasmid containing the chaperones was co-transformed with huPON1 fused with thioredoxin into BL21(DE3) electrocompetent cells (0.5 μL of chaperone, 0.5 μL huPON1 from minipreps added to BL21(DE3). The cells were recovered and plated on LB Amp/Cm. 100mL expressions in 2YT with 50 μg/mL amp and 20 μg/mL cm along with 0.5 mg/mL L-arabinose or 1 ng/mL tetracycline added accordingly for each chaperone expression system. The expressions were induced with isopropyl-β-D-thiogalactopyranoside (IPTG) at a 0.1 mM final concentration after the log phase was reached (0.8 absorbance at 600 nm) and placed at 30 °C for 4 hrs. Cells were then pelleted by centrifugation and placed at –80°C. Frozen pellets were then resuspended in 6mL lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$, 10% Glycerol, 0.1% Tergitol) with DTT (0.1 mM final concentration). Then, the cells were passed through a syringe and sonicated. Next, 10% tergitol (0.1% final concentration) was added and placed on a rotating mixer for 2.5 hours. The cells were pelleted by centrifugation at 20,000 rpm for 30 minutes. Ni-

NTA agarose (0.5 mL of 50 percent) was equilibrated with lysis buffer and added to supernatant. The exact same procedure was followed from table 2 for purification. Again, paraoxon activity assays were done with purified huPON1 co-expressed with each chaperone plasmid. Purified huPON1 was confirmed on 12.5% SDS-PAGE gels.

Initial experiments confirmed the chaperone system of choice to be pKJE7 (DnaK/DnaJ/GrpE) for co-expression with huPON1. We have also co-expressed huPON1 variants that have increased solubility and activity with pKJE7. Variants of huPON1 (obtained from M. Sarkar/G. Matic) included an N-terminal deletion of huPON1, HDL-huPON1 (mutations within HDL binding site of huPON1), N-terminal deletion HDL-huPON1. Each variant is tagged with MBP. Wild-type huPON1 with MBP was also included with these experiments. The same procedure was followed as above for co-expression in BL21(DE3). However, 1 L 2YT expressions were used as opposed to 100 mL. Frozen pellets were then resuspended in 25 mL lysis buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM $CaCl_2$, 10% Glycerol, 0.1% Tergitol) with DTT (0.5 mM final concentration). Each of the following steps were identical to the procedure described in the first chaperone experiment. For purification, table 3 shows the protocol used (M. Sarkar).

Table 3. *Purification Buffers for huPON1 variants co-expressed with KJE7*

Each buffer contains 20mM Tris-HCl pH 8.0					
Buffer Name	NaCl	$CaCl_2$	Tween	Imidazole	Amount added
Wash 1	50 mM	1 mM	None	40 mM	20 mL
Wash 2	1 M	1 mM	None	None	10 mL
Wash 3	500 mM	1 mM	None	None	10 mL
Wash 4	250 mM	1 mM	None	None	10 mL
Wash 5	None	1 mM	None	None	1 mL
Elution 1	150 mM	1 mM	0.1%	125 mM	5 mL

Paraoxon activity assays were done with purified huPON1 variants co-expressed with pKJE7. Purified huPON1 variants were confirmed on 12.5% SDS-PAGE gels.

Results and Discussion

Expression and transport of G2E6 and huPON1 into periplasm

Figure 8 represents the 12.5% SDS-PAGE gel of G2E6 fusion protein (82 kDa) expression from the periplasmic vector. All boxed bands on the following SDS-PAGE gels should represent the target protein based on the protein marker and previous experiments. Figures 9 and 10 are activity assays done against paraoxon. Both the protein gel and activity assays were from the smaller scaled experiment (100 mL) that included lysozyme in the resuspension buffer. The conclusion is that the MBP-G2E6 fusion protein was being expressed (seen on gel). However, the activity assays demonstrate insufficient transport of the fusion protein because 80 to 90 percent of the active protein is present in the sonicated fraction. There is minimal activity in the osmotic shock fluid representing the periplasmic protein.

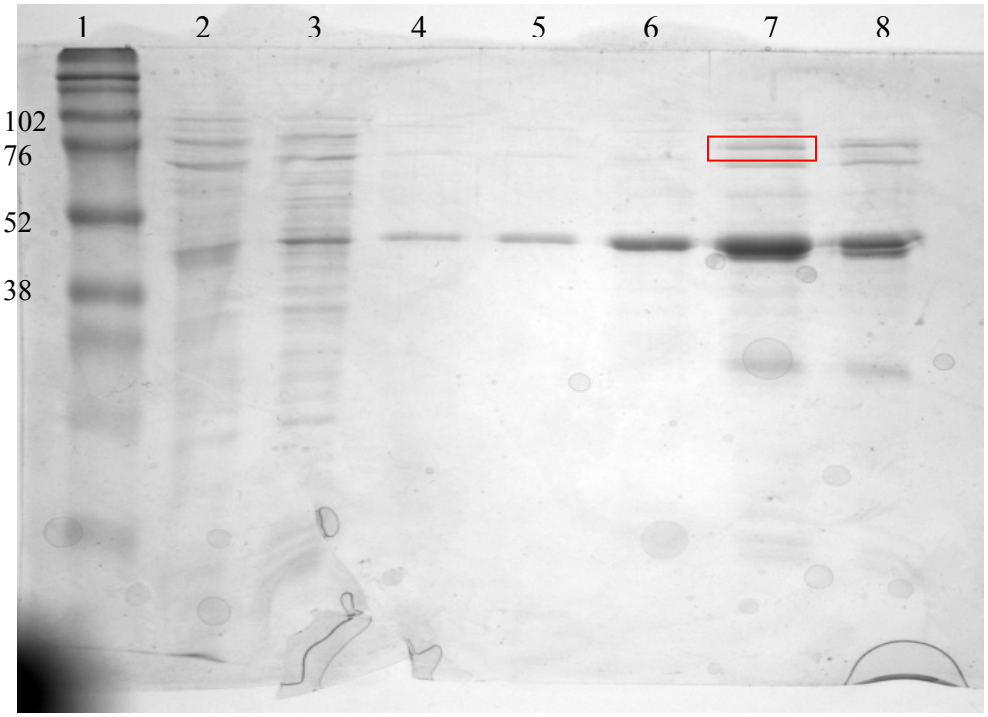


Figure 8. 12.5% SDS-PAGE gel showing MBP-G2E6 fusion protein (82 kDa) from periplasmic expression (100 mL). Lane 1-8 in the following order are osmotic shock fluid, flow-through, wash one, wash two, wash three, elution one, and elution two. The highlighted band is our protein, MBP-G2E6 (82 kDa) from elution one. The larger band beneath 52 kDa is thought to be MBP (42 kDa).

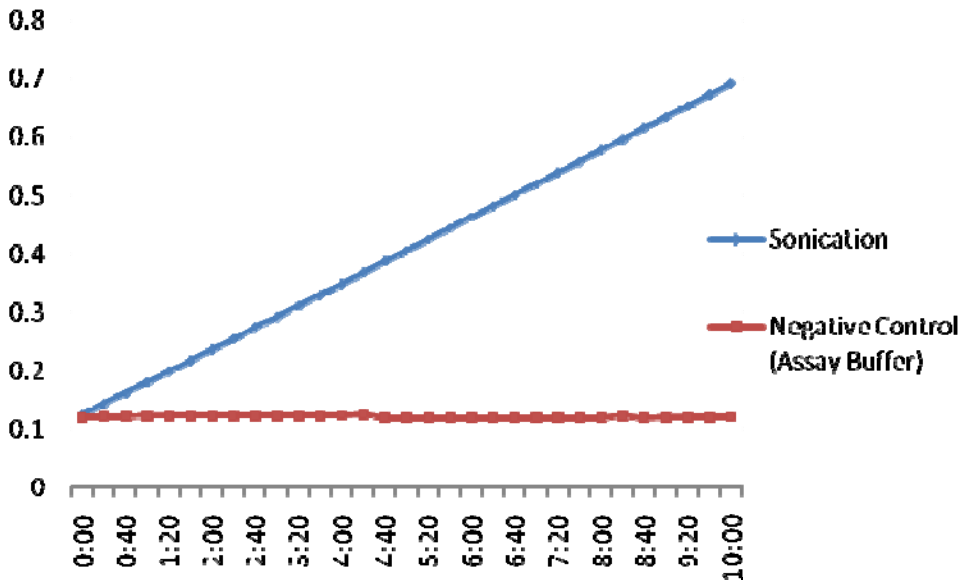


Figure 9. Paraoxon activity assay of sonicated fraction of MBP-G2E6 from periplasmic expression (100 mL). MBP-G2E6 shows activity against paraoxon.

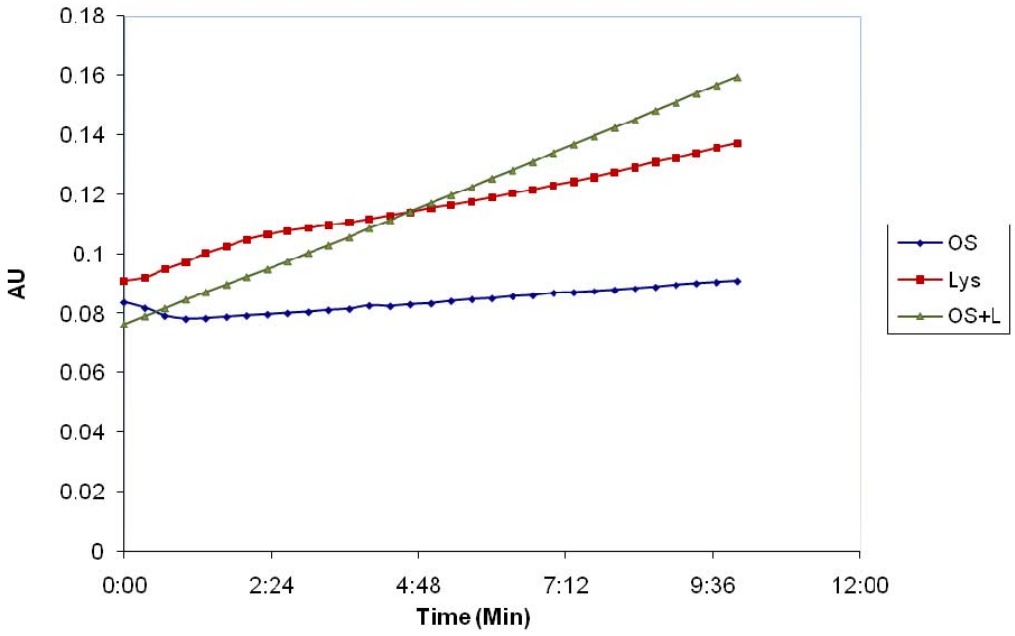


Figure 10. Paraoxon activity assay of MBP-G2E6 from osmotic shock fluid in the periplasmic expression (100 mL). The following symbols are OS-osmotic shock fluid, Lys-lysozyme, and OS+L- osmotic shock fluid with lysozyme. The most active MBP-G2E6 protein came from the recovery using osmotic shock with lysozyme treatment. Still, these elutions showed much less activity than the sonciated fraction.

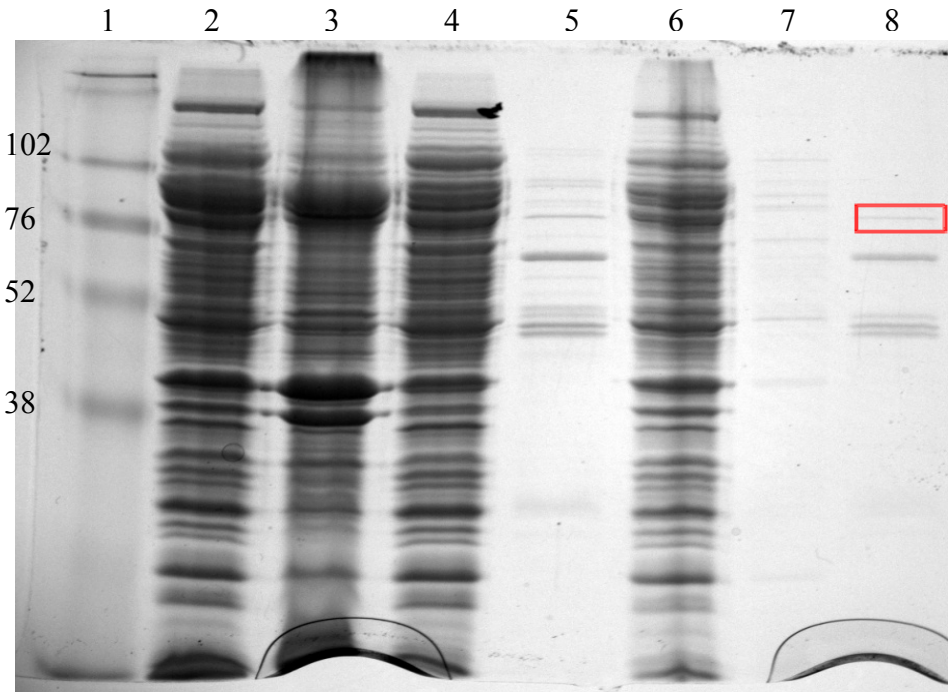


Figure 11. 12.5% SDS-PAGE gel representing purified huPON1 from periplasmic expression (100 mL). Lane 1 is the marker. Lane 2 is the total fraction of protein. Lanes 3-5 are part of the sonication and in order are the pelleted fraction, soluble fraction, and eluted fraction. Lanes 6-8 are part of the osmotic shock fluid and in order are the pellet, soluble fraction, and the eluted fraction. As shown, there is a minimal amount of protein in the osmotic shock fluid in comparison to the sonicated fraction. This again proves insufficient transportation of MBP-huPON1 to the periplasm.

The periplasmic expressions of MBP-G2E6 and MBP-huPON1 showed there was protein being expressed within the cytoplasm. However, whether or not the signal sequence was compatible or the protein was able to cross the membrane to the periplasm is unknown.

Increase solubility and activity of huPON1 in E. coli through pKJE7

The initial co-expression with the Takara chaperone systems confirmed that pKJE7 (DnaK/DnaJ/GrpE) was benefiting the solubility of huPON1, resulting in enough protein to maintain activity against paraoxon. Figure 12 shows pGro7, pKJE7, and pTf16 co-expressed with huPON1-trx in BL21(DE3).

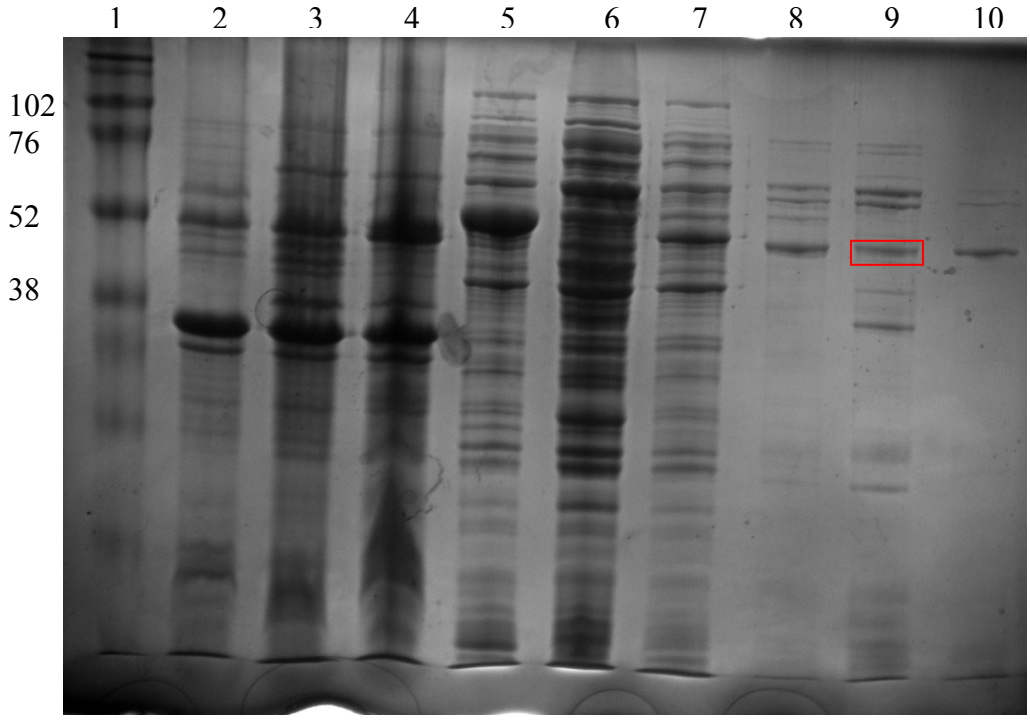


Figure 12. 12.5% SDS-PAGE gel representing pGro7, pKJE7, and pTf-16 co-expressed with huPON1-trx (57 kDa). The first three lanes represent pelleted fraction, followed by lysate, and finally eluted protein (order of pGro7, pKJE7, and pTf16 for pelleted fraction- lanes 2-4, and same for lysates and elutions). Eluted huPON1 (with pKJE7) is highlighted. Note: huPON1-trx (57 kDa) does run low on SDS-PAGE gels based from previous experiments (~50 kDa).

Figure 13 shows there is activity against paraoxon when huPON1-trx co-expressed with pKJE7. Figure 14 confirms the initial results of active huPON1 co-expressed with pKJE7 against paraoxon (V.Shete). pG-KJE8 and pG-Tf2 are not shown in a gel or activity assay because neither gave sufficient amounts of solubilized or active protein.

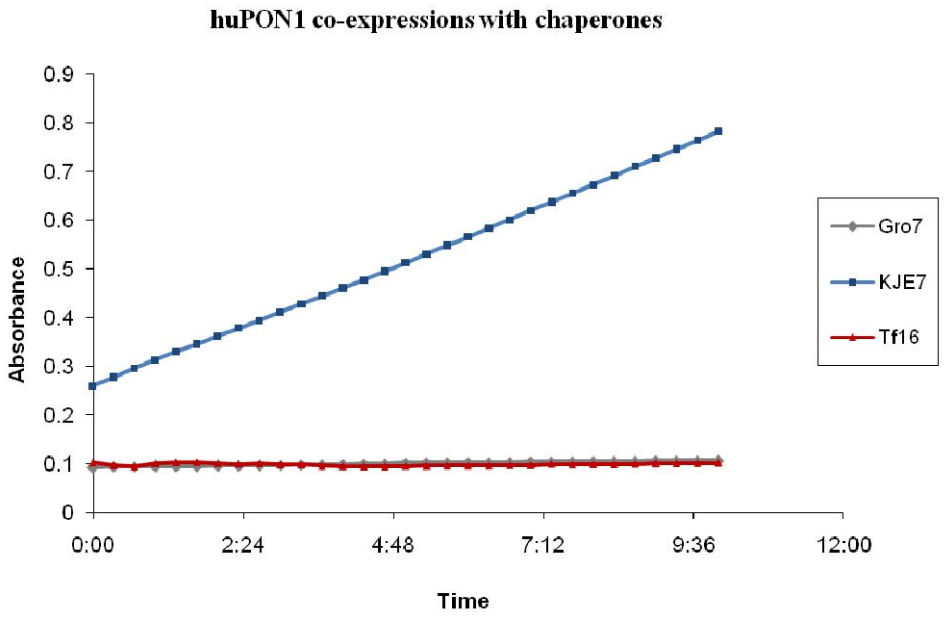


Figure 13. Paraoxon activity assay of huPON1-trx co-expressed with pGro7, pKJE7, and pTf16. Both pGro7 and pTf16 co-expressed with huPON1-trx do not show any activity. However, huPON1-trx co-expressed with pKJE7 (DnaK, DnaJ, GrpE) does show activity against paraoxon.

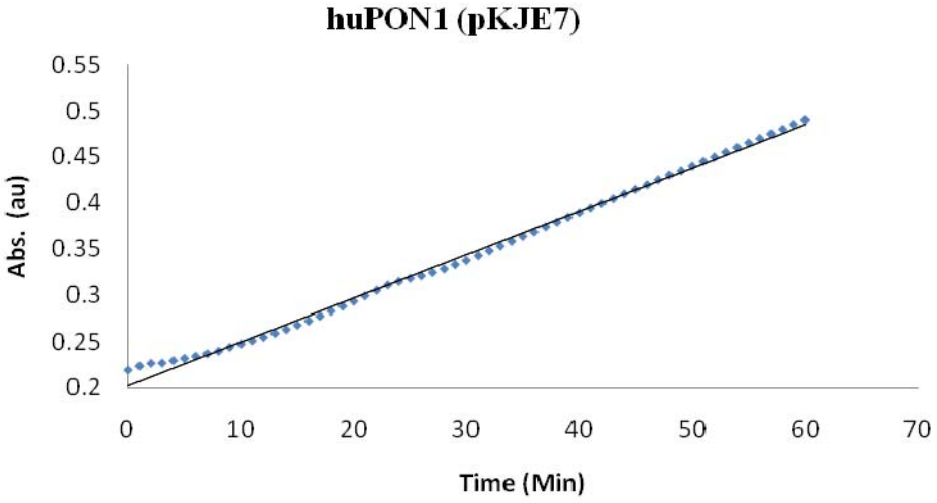


Figure 14. Paraoxon activity assay against huPON1-trx co-expressed with pKJE7. This experiment confirmed activity against paraoxon.

Co-expression of pKJE7 with huPON1 variants

The next step after selecting pKJE7 was to select variants of huPON1 to co-express with pKJE7. The engineered huPON1 variants selected had already shown increased solubility in comparison to wild-type huPON1 (Magliery, Sarkar, Matic). However, there had been a challenge in the purification steps of these variants. The purification protocol from Table 3 (M.Sarkar) was the newest and best procedure for variant purification. Yet, the purified protein still contained apparent truncation products that had been eluted with it. The gels below show some other bands, but the bands for N-terminal deletion huPON1 and N-terminal deletion HDL-huPON1 have very distinct, clean bands. Nonetheless, each purified protein exhibited cleaner bands with pKJE7 than without. Figure 15 is presents purified huPON1-MBP with and without DnaK/DnaJ/GrpE for reference. Figure 16 shows HDL-huPON1 and N-terminal deletion HDL-huPON1 co-expressed with pKJE7. Figure 17 shows wild-type huPON1 and N-terminal deletion huPON1 co-expressed with pKJE7. Figure 18 proves that all variants co-expressed with pKJE7 proved to be active with N-teal deletion HDL-huPON1 being the most active.

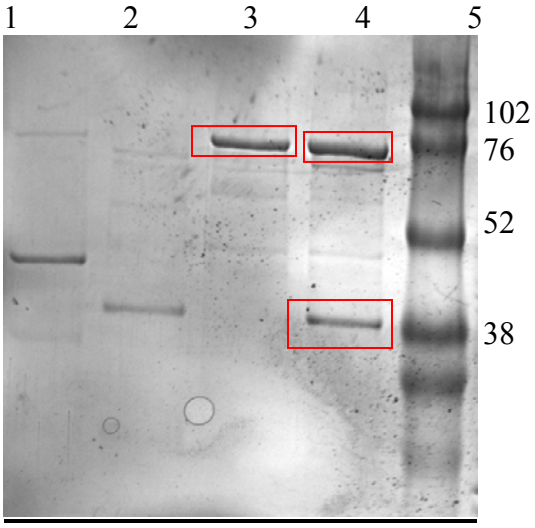


Figure 15. SDS-PAGE gel showing reference band for huPON1-MBP (~82 kDa). It is important to note huPON1-MBP does run low (~75-76 kDa). All lanes represent purified protein from eluted fractions. Lane 1 is origami B, lane 2 is DnaK/DnaJ/GrpE, lane 3 is huPON1-MBP, and lane 4 is huPON1-MBP co-expressed with DnaK/DnaJ/GrpE. The lower band boxed in lane 4 is DnaJ (40 kDa).

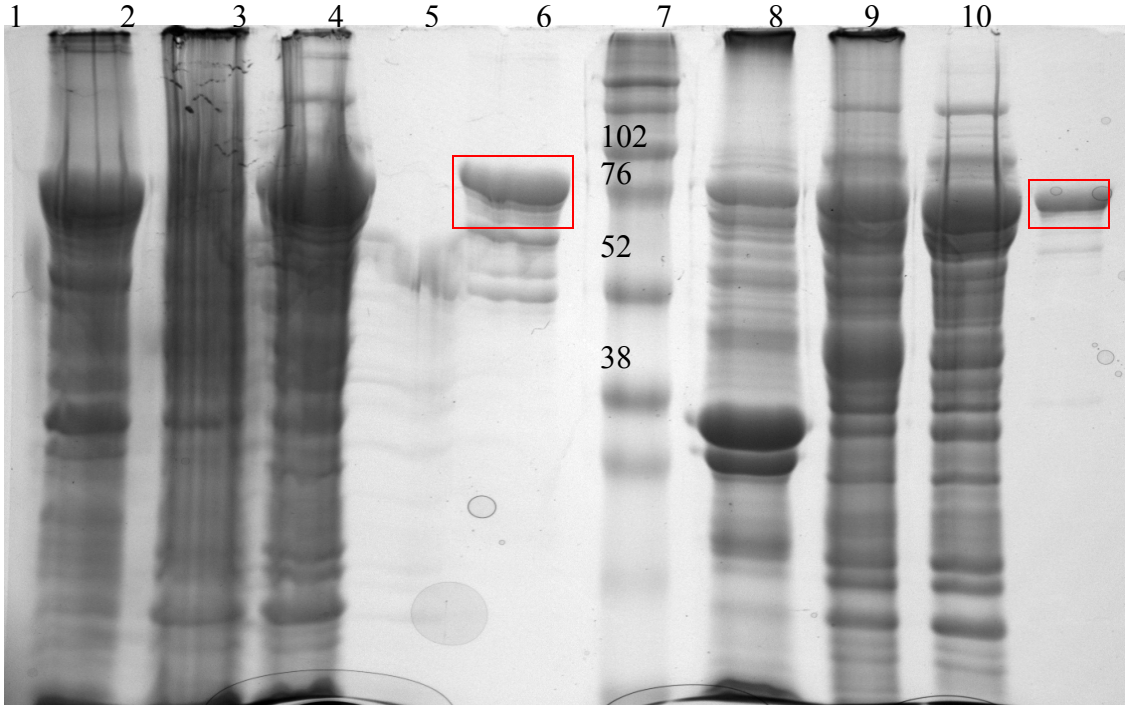


Figure 16. 12.5% SDS-PAGE gel showing HDL-huPON1 and N-terminal deletion HDL-huPON1 co-expressed with pKJE7. Lanes 1-5 are HDL-huPON1 and from left to right are pellet, supernatant, flow-through, wash 5, and elution 1. Lane 6 is the marker. Lanes 7-10 are N-terminal deletion HDL-huPON1 and from left to right are pellet, supernatant, flow-through and elution. N-terminal deletion HDL-huPON1 co-expressed with pKJE7 shows a very clean band.

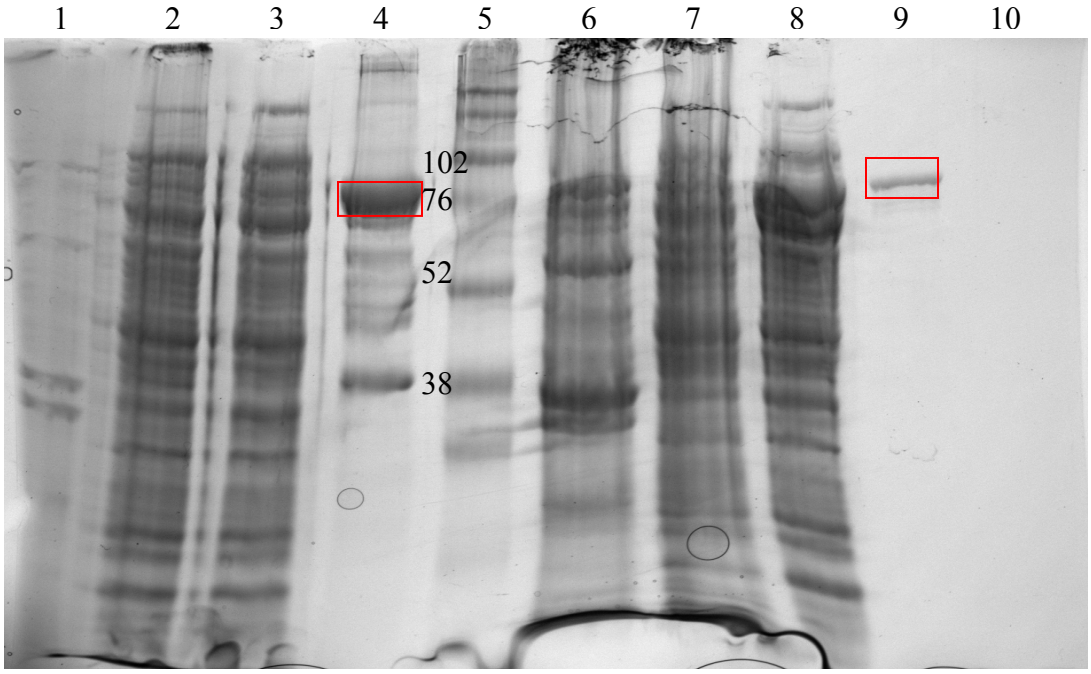


Figure 17. 12.5% SDS-PAGE gel with wild-type huPON1 and N-terminal deletion huPON1 co-expressed with pKJE7. Lanes 1-4 are wild-type huPON1 and from left to right are pellet, supernatant, flow-through, and elution 1. Lane 5 is the marker. Lanes 6-10 are N-terminal deletion huPON1 and from left to right are pellet, supernatant, flow-through, elution 1 and wash 5. Wild-type huPON1 co-expressed with pKJE7 shows a large amount of protein. Although, N-terminal deletion huPON1 shows less protein, it has a much cleaner band.

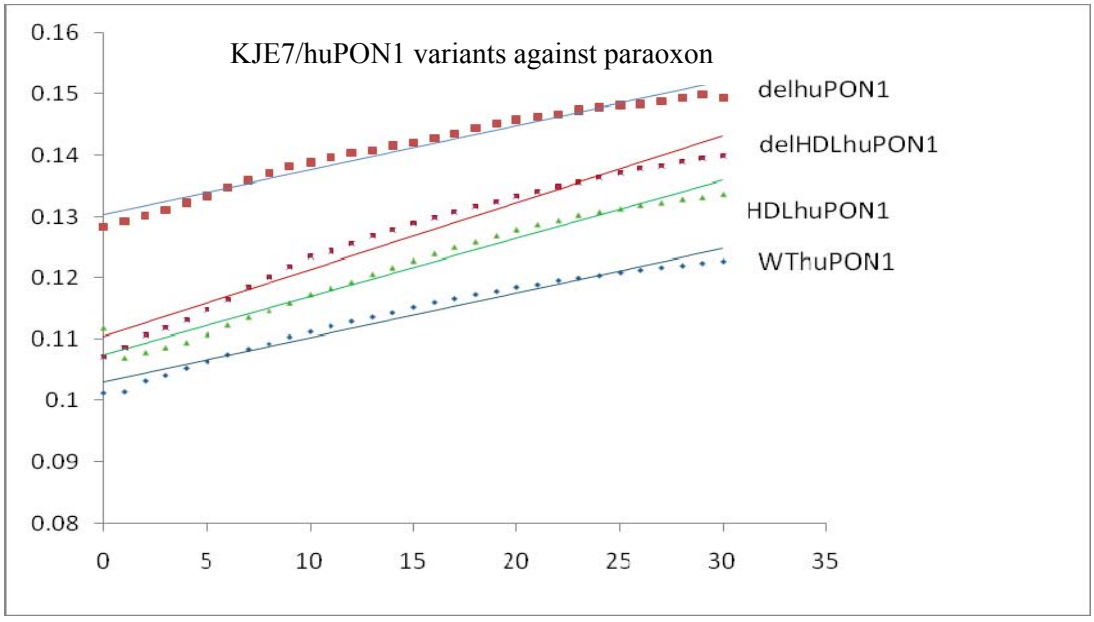


Figure 18. Activity assay against paraoxon of huPON1 variants co-expressed with pKJE7. While all variants are active when co-expressed with pKJE7, N-terminal deletion HDL-huPON1 shows the highest level of activity against paraoxon.

Remarks

The periplasmic experiments did not successfully transport much of the target protein. The periplasmic expression vector was completed and its sequencing was confirmed. There are several explanations that could explain why this problem arose. First, it could have been the signal sequence being cleaved before the protein intermediate was able to reach the inner membrane. Moreover, there are other possible signal sequences that could have replaced the initial one. Another possible problem could have been that the sequence became attached or unable to cross over the membrane and reach the periplasm. This could have been due to the HDL-binding site located on huPON1.

All five chaperone plasmids from Takara Bio Inc. were tested and co-expressed with huPON1. KJE7 co-expression has given active huPON1 along with cleaner more homogenous protein in comparison to non co-expressed huPON1. We are now pursuing the expression of unfused huPON1 vector to see if co-expression with KJE7 is able to again produce an active huPON1 without MBP or trx fusions. The cloning was done using ligation-independent cloning.[22] Cloning has been completed and expression and purification will be done in the upcoming weeks.

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